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Purification and Properties of Biliverdin Reductases from Pig Spleen and Rat Liver[1]

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Biliverdin reductase was purified from pig spleen soluble fraction to a purity of more than 90% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was a monomer protein with a molecular weight of about 34,000. Its isoelectric point was at 6.1–6.2. The enzyme was strictly specific to biliverdin and no other oxidoreductase activities could be detected in the purified enzyme preparation. The purified enzyme could utilize both NADPH and NADH as electron donors for the reduction of biliverdin. However, there were considerable differences in the kinetic properties of the NADPH-dependent and the NADH-dependent biliverdin reductase activities: K_m for NADPH was below 5 μM while that for NADH was 1.5–2 mM; the pH optimum of the reaction with NADPH was 8.5 whereas that of the reaction with NADH was 6.9; K_m for biliverdin in the NADPH system was 0.3 μM whereas that in the NADH system was 1–2 μM. In addition, both the NADPH-dependent and NADH-dependent activities were inhibited by excess biliverdin, but this inhibition was far more pronounced in the NADPH system than in the NADH system. IXα-biliverdin was the most effective substrate among the four biliverdin isomers, and the dimethylester of IXα-biliverdin could not serve as a substrate. Biliverdin reductase was also purified about 300-fold from rat liver soluble fraction. The hepatic enzyme was also a monomer protein with a molecular weight of 34,000 and showed properties quite similar to those of the splenic enzyme as regards the biliverdin reductase reaction. The isoelectric point of the hepatic enzyme, however, was about 5.4. It was assumed that NADPH rather than NADH is the physiological electron donor in the intracellular reduction of IXα-biliverdin. The stimulatory effects of bovine and human serum albumins on the biliverdin reductase reactions were also examined.

Biliverdin is the final product of physiological heme degradation in mammalian tissues; this process is thought to be catalyzed by the microsomal heme oxygenase (*1–6*), and biliverdin is then reduced to bilirubin by biliverdin reductase [EC 1.3.1.24], consuming reduced pyridine nucleotides (*7*). Despite the importance of biliverdin reductase in linear tetrapyrrole metabolism, the molecular

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and catalytic properties of this enzyme have not been well elucidated. In 1965 Singleton and Laster (*8*) obtained a 15-fold purified preparation of biliverdin reductase from guinea pig liver soluble fraction and reported that the enzyme was more active with NADH than NADPH. However, Tenhunen *et al.* (*9*) reported in 1970 that biliverdin reductase partially purified (52-fold) from rat liver soluble fraction had an absolute and stoichiometric requirement for NADPH. Subsequently Colleran and O'Carra (*10, 11*) indicated that guinea pig liver extracts contained both NADH-dependent and NADPH-dependent activities and that these activities were due to a single enzyme system capable of utilizing both NADH and NADPH. They also found that biliverdin reductase was essentially specific to IXα-biliverdin; the IXβ- and IXδ-isomers were very poor substrates and the IXγ-isomer did not serve as a substrate. IXα-biliverdin reduction was markedly inhibited by excess biliverdin. These studies by Colleran and O'Carra provided information on the general features of the biliverdin reductase reaction. However, to clarify further the nature of biliverdin reductase as well as the properties of the reaction catalyzed by this enzyme, it is essential to have a highly purified preparation of this enzyme. Previous efforts to purify this enzyme have not been wholly successful.

In the present report, we describe the purification of biliverdin reductase, as well as the properties of the biliverdin reductase reaction catalyzed by the purified enzyme preparation.

MATERIALS AND METHODS

Materials—Fresh pig spleens used as a source of splenic biliverdin reductase were obtained at a local slaughterhouse. The hepatic biliverdin reductase was prepared from the livers of Wistar rats, 120–150 g body weight.

Reagents and biochemicals were obtained from the following sources: Sephadex G-100, Sephadex G-200, and QAE-Sephadex, from Pharmacia Fine Chemicals, Uppsala; CM-cellulose (CM-23) and DEAE-cellulose (DE-32), from Whatman Biochemicals, Maidstone; Wakogel B-10, Wakogel C-200, human serum albumin, from Wako Pure Chemical Industries, Osaka; crystalline bilirubin, chlorohemin, bovine serum albumin, FMN, yeast alcohol dehydrogenase, ovalbumin, and horse heart myoglobin, from Sigma Chemical Company, St. Louis; pig heart malate dehydrogenase, chymotrypsinogen A, ox liver catalase, and rabbit muscle lactate dehydrogenase, from Boehringer Mannheim-Yamanouchi, Tokyo; pyridine nucleotides, from Oriental Yeast Company, Tokyo; FAD, from Wakamoto Pharmaceutical Company, Tokyo; Ampholine, from LKB Produkter, Bromma. Other reagents were also obtained commercially. Hydroxyapatite was prepared according to the method described by Bernardi (*12*).

IXα-biliverdin was prepared by the oxidation of crystalline bilirubin with ferric chloride in boiling glacial acetic acid essentially according to the method described by Lemberg (*13*), and pure biliverdin-IXα was isolated from the mixture of oxidation products of bilirubin by silica gel column chromatography, instead of by crystallization. Namely, the amorphous powder of crude biliverdin obtained from 0.5 g of crystalline IXα-bilirubin by the procedures described by Lemberg was divided into two equal portions. Each portion was suspended in a small volume of pyridine (about 10 ml) and applied to a silica gel column (3.6×27 cm) (Wakogel C-200) in chloroform–acetic acid (97 : 3). On elution with the same solution, a small amount of bilirubin that remained unoxidized was eluted first, then unidentified brown and reddish purple pigments were eluted successively. When these pigments were completely eluted from the column, IXα-biliverdin (clear dark green color) was eluted with a slightly more polar eluant (chloroform–methanol–acetic acid, 92 : 5 : 3). Further elution with more polar eluants (chloroform–methanol–acetic acid, 77 : 20 : 3 and methanol–acetic acid, 97 : 3) yielded other unidentified greenish-brown and brown pigments. Of the fractions eluted with the second eluant, those which gave only one spot on analytical thin layer chromatography were collected. The analytical thin layer chromatography was carried out on 20×5 cm plates precoated with 0.25 mm layers of Wakogel B-10 activated at 150°C for 3 h. The pure biliverdin obtained above gave a single spot with an R_f value of 0.25–0.27 when developed with chloroform–methyl alcohol–acetic acid (94 : 5 : 1).

The biliverdin preparations obtained from the two silica gel columns were combined and the

solvent was evaporated off under reduced pressure at 30–40°C. The pigment was redissolved in 1% NaOH and precipitated by adding 1% acetic acid. The precipitate was collected by centrifugation, washed several times with distilled water to remove salts, then freeze-dried. The biliverdin powder obtained was stored at 4°C in the dark. From 0.5 g of crystalline bilirubin, 30–50 mg of pure IXα-biliverdin was usually obtained.

The purity of biliverdin prepared by this method was confirmed in various systems of analytical thin layer chromatography (*14–16*) and from the absorption spectrum, using crystalline IXα-biliverdin (kindly provided by Dr. McDonagh) and its dimethyl ester as standards. The pure biliverdin preparation showed two absorption maxima at 384 nm and 670 nm with millimolar extinction coefficients of 49.0 and 15.0, respectively, in potassium phosphate buffer (pH 7.4) (*cf.* Fig. 7).

For daily use, 2–3 mg of the purified biliverdin was dissolved in about 0.3 ml of 0.1 N KOH and diluted with 0.1 M potassium phosphate buffer (pH 7.4) to approximately 0.2 mM. The precise concentration of biliverdin in the solution was estimated on the basis of the absorbance at 670 nm.

Four biliverdin isomers were prepared from the products (verdohemochrome) of the coupled oxidation of pyridine hemin with ascorbic acid as the dimethyl esters, according to the procedure of Bonnet and McDonagh (*14*). Each isomer was further purified by thin layer chromatography according to the methods of O'Carra and Colleran (*15*). The dimethyl ester of each isomer was converted to the free acid by incubation with 1% KOH at 37°C for 2 h in the dark. The mixture was then neutralized with acetic acid and the acid form of biliverdin was extracted with chloroform. Chloroform was evaporated off and the pigment was dissolved in 0.1 N KOH, then diluted with 0.1 M potassium phosphate buffer (pH 7.4) as described above.

Estimation of Molecular Weight by Gel Filtration and by Sodium Dodecyl Sulfate (SDS)-Polyacrylamide Gel Electrophoresis—Estimation of the molecular weight of biliverdin reductase by gel filtration was performed according to the method of Andrews (*17*) with a column (2.5 × 100 cm) of Sephadex G-200 previously equilibrated with 50 mM potassium phosphate buffer (pH 7.4) and calibrated with the following proteins (molecular weights in parentheses): yeast alcohol dehydrogenase (150,000), pig heart malate dehydrogenase (67,000), ovalbumin (45,000), and chymotrypsinogen A (25,000). Electrophoresis on SDS-polyacrylamide gel was performed as described by Weber and Osborn (*18*). Calibration proteins and their molecular weights were: catalase (58,000), ovalbumin (45,000), lactate dehydrogenase (36,000), chymotrypsinogen A (25,000), and horse heart myoglobin (17,200). To assess the purity of the biliverdin reductase preparation, the gel was stained with Coomassie blue and the relative optical densities of the protein bands on the gel were measured with a Hitachi gel scanner, recording the difference in absorbances at 550 nm and 400 nm.

Determination of Protein—Protein was determined by the method of Lowry *et al.* (*19*), using bovine serum albumin as a standard.

Assay of Biliverdin Reductase Activity—The reaction was conducted in a cuvette placed in a constant temperature chamber at 37°C attached to a Hitachi 200-20 double-beam spectrophotometer, and the enzymic conversion of biliverdin to bilirubin was monitored in terms of the increase in absorbance at 468 nm. The standard assay mixture contained, in a final volume of 2 ml, 0.1 M potassium phosphate buffer (pH 7.4), 10 μM IXα-biliverdin, 1 mg/ml bovine serum albumin, 100 μM NADPH (or 2 mM NADH), and an appropriate amount of the enzyme preparation. The concentration of NADH used in the standard assay system was lower than the saturating concentration (K_m for NADH was 1.5–2.0 mM, as described later), but the NADH-dependent biliverdin reductase activity could be adequately detected at this NADH concentration. Reduced pyridine nucleotide was omitted in the reference cuvette. The reaction was started by the addition of reduced pyridine nucleotide, after preincubation for 5 min at 37°C. Formation of bilirubin was calculated by assuming that the difference in the millimolar extinction coefficients of bilirubin and biliverdin at 468 nm is 46.0: the millimolar extinction coefficients of bilirubin and biliverdin at 468 nm were 52.0 and 6.0, respectively, under the experimental conditions employed.

As shown in Fig. 1, however, the time course of the NADPH-dependent reaction displayed a sigmoidal curve, whereas the reaction with NADH did not. This was true for the reactions with

either the pig spleen enzyme or the rat liver enzyme. Therefore, to express the enzyme activity, we defined one unit of biliverdin reductase as the amount of the enzyme which catalyzed the reduction of half of 20 nmol of biliverdin in one min under the standard assay conditions; since the concentration of biliverdin in the standard reaction mixture was 10 μM, the enzyme units correspond to the reciprocals of the time in minutes required to obtain an increase of absorbance of 0.23 at 468 nm, as indicated by the dashed lines in Fig. 1. The enzyme units thus defined were linearly proportional to the amounts of enzyme protein used in both the NADPH-dependent and NADH-dependent reactions, as shown in the inset in Fig. 1. Therefore, this measure was employed in most of the experiments in the present study, although other measures were also employed in some experiments for convenience, as indicated in the appropriate tables and figures.

A Hitachi 323 recording spectrophotometer was used in some experiments.

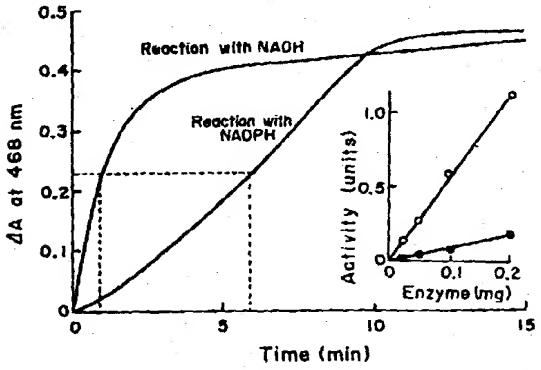

Fig. 1. Time courses of the NADPH-dependent and NADH-dependent biliverdin reductase reactions under the standard assay conditions. The reaction mixture, containing 1.06 mg protein of the CM-cellulose fraction of splenic biliverdin reductase, was preincubated for 5 min at 37°C and the reaction was started by adding NADPH (final 100 μM) or NADH (final 2 mM). The increase of absorbance at 468 nm was recorded. *Inset:* The relationship between the activity units and the amount of enzyme. ●, NADPH-dependent reaction; ○, NADH-dependent reaction.

RESULTS

Purification of Biliverdin Reductase from Pig Spleen—Fresh pig spleens (1.2 kg) were homogenized in 0.02 M potassium phosphate buffer (pH 7.4) containing 0.134 M KCl with a Waring blender to give a 20% homogenate. The homogenate was centrifuged succesively at 8,000 × g for 15 min and at 56,000 × g for 2 h, and the supernatant obtained was used as the starting extract. The results of a typical purification experiment are shown in Table I.

TABLE I. Summary of the purification of biliverdin reductase from pig spleen. The procedures are described in detail in the text.

Fraction	Total protein (mg)	Biliverdin reductase activity				Ratio of activities measured with NADH and with NADPH
		With NADPH (100 μM)		With NADH (2 mM)		
		Specific activity (unit/mg)	Total activity (unit)	Specific activity (unit/mg)	Total activity (unit)	
56,000 × g supernatant	66,300	0.0349	2,310	0.298	19,700	8.5
Solid ammonium sulfate (35–70%)	27,600	0.107	2,950	0.646	17,800	6.0
CM-cellulose	20,700	0.104	2,150	0.606	12,500	5.8
1st Hydroxyapatite	8,770	0.240	2,100	1.46	12,800	6.1
1st Sephadex G-100	876	1.06	929	7.19	6,300	6.8
DEAE-cellulose	76.6	8.86	679	61.6	4,720	7.0
2nd Hydroxyapatite	41.0	12.4	508	86.1	3,530	7.0
Electrofocusing	—	—	150	—	1,050	7.0
2nd Sephadex G-100	2.54	23.0	58.4	161	409	7.0
3rd Sephadex G-100	0.900	24.0	21.6	160	144	6.7

Ammonium sulfate fractionation: The 56,000 × *g* supernatant was fractionated with solid ammonium sulfate between 0.35 and 0.7 saturations and the precipitate obtained was suspended in 1 mM potassium phosphate buffer (pH 6.5) then dialyzed against 100 volumes of the same buffer for 6 h (solid ammonium sulfate fraction).

CM-cellulose column chromatography: The solid ammonium sulfate fraction (about 500 ml) was passed through a column of CM-cellulose (5 × 30 cm) previously equilibrated with 1 mM potassium phosphate buffer (pH 6.5). In this procedure almost all of the hemoglobin was retained on the column, whereas biliverdin reductase was not adsorbed. The column was washed with the same buffer until no further biliverdin reductase activity emerged, and the washings were combined with the flow-through (CM-cellulose fraction).

1st hydroxyapatite column chromatography: The CM-cellulose fraction was diluted with potassium phosphate buffer (pH 7.4) to reduce the protein concentration to about 5 mg/ml, then the solution was divided into five equal portions and applied separately to five hydroxyapatite columns (5 × 10 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4). Each column was washed with 100 ml of the same buffer, and biliverdin reductase was eluted with 70 mM potassium phosphate buffer (pH 7.4). The five eluates were combined (1st hydroxyapatite fraction, about 800 ml).

1st Sephadex G-100 gel filtration: Proteins in the 1st hydroxyapatite fraction were precipitated by the addition of solid ammonium sulfate (0.8 saturation), then taken up in a small volume of 20 mM potassium phosphate buffer (pH 7.4) and dialyzed against 100 volumes of the same buffer for 6 h. The dialyzed solution (about 120 ml) was divided into four equal portions and each portion was applied separately to a Sephadex G-100 column (5 × 100 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4). Elution was performed with the same buffer and the active fractions from the four columns were pooled (1st Sephadex G-100 fraction).

DEAE-cellulose column chromatography: The 1st Sephadex G-100 fraction was applied to a column of DEAE-cellulose (2.5 × 45 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4). Elution was performed with 200 ml of the same buffer, followed by a linear gradient formed from 500 ml of the starting buffer and 500 ml of 250 mM potassium phosphate buffer (pH 7.4). The enzyme activity emerged in the early portion of the gradient and the active fractions were combined (DEAE-cellulose fraction).

2nd hydroxyapatite column chromatography: The DEAE-cellulose fraction was diluted with distilled water to reduce the buffer concentration to 10 mM, then applied to a column of hydroxyapatite (2.5 × 10 cm) previously equilibrated with 10 mM potassium phosphate buffer (pH 7.4). Proteins were eluted from the column first with 100 ml of the same buffer, then with a linear gradient formed from 200 ml of the starting buffer and 200 ml of 150 mM potassium phosphate buffer (pH 7.4). The enzyme-active fractions were combined (2nd hydroxyapatite fraction).

Preparative electrofocusing: The 2nd hydroxyapatite fraction was concentrated to about 15 ml by ultrafiltration and subjected to preparative electrofocusing in the pH range of 5 to 7, using an LKB electrofocusing column of 110 ml capacity operated according to the manufacture's instructions. After running at 600 volts for 45 h, the content of the column was pumped out at a rate of 2 ml per min and fractions of 2.3 ml were collected. The fractions between pH 5.9 and 6.5 were combined (electrofocusing fraction). The isoelectric point of biliverdin reductase from pig spleen was estimated to be 6.1–6.2.

2nd Sephadex G-100 gel filtration: The electrofocusing fraction was applied to a column of Sephadex G-100 (5 × 100 cm) equilibrated with 20 mM potassium phosphate buffer (pH 7.4) and proteins were eluted with the same buffer. The active fractions were combined (2nd Sephadex G-100 fraction).

3rd Sephadex G-100 fraction: The 2nd Sephadex G-100 fraction was concentrated to about 5 ml by ultrafiltration and was further fractionated on a column of Sephadex G-100 (2.5 × 100 cm), using 50 mM potassium phosphate buffer (pH 7.4). The active fractions were pooled (3rd Sephadex G-100 fraction).

The purified enzyme preparation in 50 mM potassium phosphate buffer (pH 7.4) could be stored at −20°C for several months without loss of activity.

It should be noted (Table I) that biliverdin

reductase was active with both NADH and NADPH, and the ratio of the activities with NADH and NADPH remained nearly constant throughout the purification procedure; in all instances the activities obtained with NADH were about 7 times higher than those obtained with NADPH under the assay conditions employed.

Molecular Properties of the Splenic Biliverdin Reductase—The purified biliverdin reductase preparation finally obtained gave a distinct major protein band when subjected to SDS-polyacrylamide gel electrophoresis (Fig. 2), and its molecular weight was estimated to be about 34,000. In addition to the major band, one or two faint bands were occasionally seen on the gel: the apparent molecular weights of these additional proteins were in the range of 50,000 to 70,000 and their relative densities varied from preparation to preparation. The major protein detected on the SDS-polyacrylamide gel usually constituted 90% or more of the total protein loaded as determined with a gel scanner. The purified biliverdin reductase preparation also gave a molecular weight of 34,000 as estimated by gel filtration on a Sephadex G-200 column (data not shown). Therefore, we conclude that biliverdin reductase from pig spleen is a monomer protein with an apparent molecular weight of 34,000.

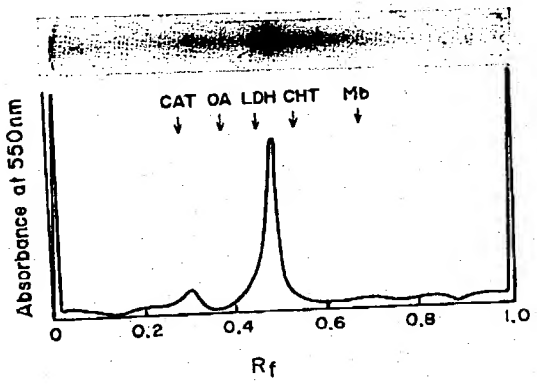

Fig. 2. Estimation of the molecular weight of the purified splenic biliverdin reductase by SDS-polyacrylamide gel electrophoresis. The purified biliverdin reductase preparation (about 6 μg protein) was loaded onto a 7.5% polyacrylamide gel (0.6×7 cm) containing 0.1% SDS. Coomassie blue was employed as the tracking dye. The marker proteins (indicated by arrows in the figure) were, from left to right: catalase (CAT); ovalbumin (OA); lactate dehydrogenase (LDH); chymotrypsinogen A (CHT); myoglobin (Mb).

The purified biliverdin reductase from pig spleen did not appear to contain any cofactor as judged from the absorption spectrum in the visible region, although this conclusion is not definitive, since the enzyme solutions available for spectrophotometric study were of relatively low concentration (about 50 μg/ml). The addition of FAD or FMN was rather inhibitory to the biliverdin reductase reaction.

Specificity of Splenic Biliverdin Reductase and Effects of Various Compounds on the Enzyme Activity—The purified splenic biliverdin reductase preparation did not exhibit any other pyridine nucleotide-dependent activity such as glucose dehydrogenase, alcohol dehydrogenase, aldehyde dehydrogenase, glutathione reductase or DT-diaphorase, when tested under appropriate assay conditions (data not shown). The biliverdin reductase activity was susceptible to thiol reagents; in these experiments, reaction mixtures containing 6 μg protein of the purified enzyme were treated with various thiol reagents and preincubated for 5 min at 37°C, then the reactions were started by the addition of NADPH. The activity was completely inhibited by 25 μM *p*-chloromercuribenzoate, whereas the inhibition by this reagent at 1 μM was only 15%. The activity was also inhibited by about 30% by 0.5 mM or 1.0 mM *N*-ethylmaleimide. The inhibition by 1 mM iodoacetamide or 1 mM arsenobenzoate, however, was less than 10%. The reaction was not affected by EDTA or other chelating agents.

Kinetic Properties of the Splenic Biliverdin Reductase Reaction—Although the purified biliverdin reductase was active with both NADH and NADPH, there were considerable differences between the kinetic properties of the reactions with NADH and NADPH.

pH optimum: The pH optimum of the reaction with NADH was 6.9, whereas that with NADPH was 8.5 (Fig. 3). In the following experiments, however, reactions were carried out at pH 7.4 unless otherwise stated.

Inhibition by excess biliverdin: The reactions with NADH and NADPH were inhibited by excess biliverdin and, as shown in Fig. 4, the inhibition was apparently independent of the concentration of NADPH or NADH. However, the reaction with NADPH was far more susceptible to excess biliverdin than the reaction with NADH; for

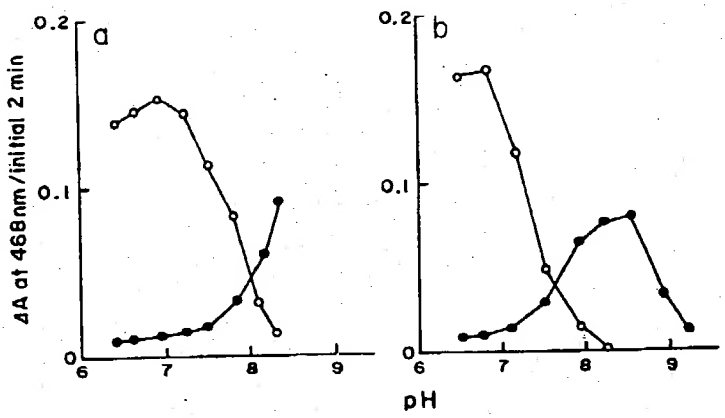

Fig. 3. The pH-activity relationships of the biliverdin reductase reactions. The reaction mixtures contained, in a final volume of 2 ml, 1.8 μg protein of purified splenic biliverdin reductase, 10 μM biliverdin, 1 mg/ml bovine serum albumin, 0.1 mM NADPH (or 2 mM NADH), and 100 mM potassium phosphate buffer in *a* or 100 mM Tris-HCl buffer in *b*. The actual pH of each assay mixture was determined at 37°C after the reaction. Other conditions were the same as the standard assay conditions. ●, NADPH-dependent reaction; ○, NADH-dependent reaction.

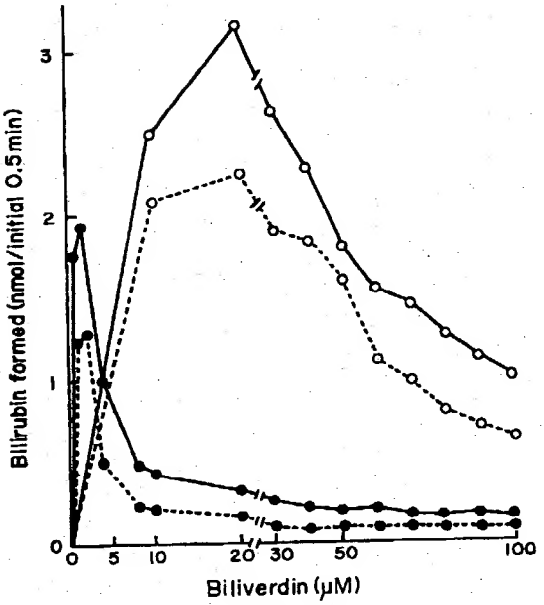

Fig. 4. Inhibition of biliverdin reductase by excess biliverdin. Each reaction mixture contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 1 mg/ml bovine serum albumin, and 3.7 μg protein of purified splenic biliverdin reductase. —●—, With 100 μM NADPH; --●--, with 10 μM NADPH; —○—, with 5 mM NADH; --○--, with 2 mM NADH. Other conditions were as described in "MATERIALS AND METHODS."

instance, the reaction with NADPH was markedly inhibited when the reaction was carried out with 10 μM biliverdin, while 10 μM biliverdin did not appear to inhibit the reaction with NADH appreciably. The data in Fig. 4 could well account for the observation (Fig. 1) that the time course of the reaction with NADPH was sigmoidal, whereas the reaction with NADH was not under the standard assay conditions with 10 μM biliverdin as a substrate.

K_m values for reduced pyridine nucleotides and biliverdin: The K_m values were determined at lower biliverdin concentrations to avoid possible substrate inhibition, and using relatively small amounts of the enzyme to ensure that the reaction was linear at least for the initial 0.5 to 1 min. Figure 5 shows double-reciprocal plots of the concentration of reduced pyridine nucleotides against the initial velocities. The plots obtained in the NADH system or the NADPH system at fixed concentrations of biliverdin as indicated in the figure crossed the axis to the left of the ordinate. This indicates that the biliverdin reductase reaction occurs by a sequential mechanism (*20*). From the data in Fig. 5, the K_m values were calculated according to Cleland (*20*); in the reaction with NADPH, K_m for biliverdin was 0.3–0.5 μM and

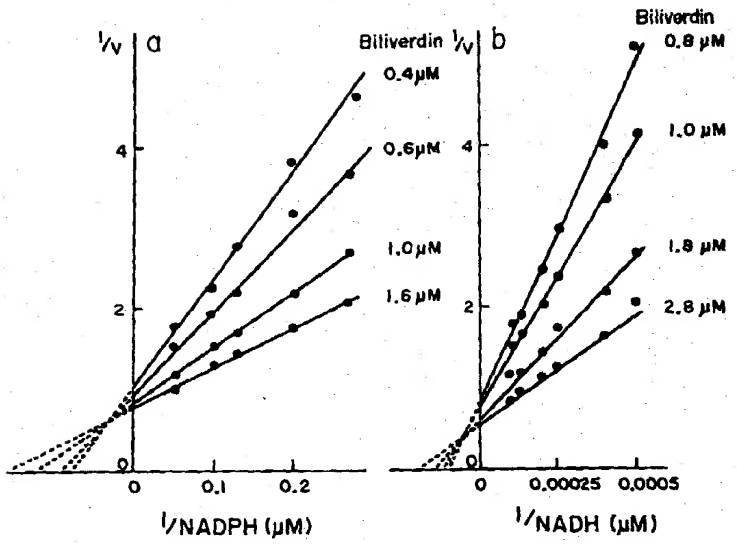

Fig. 5. The reaction rate as a function of the concentration of reduced pyridine nucleotide. The assay mixtures contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 1 mg/ml bovine serum albumin, 1.2 μg protein of purified splenic biliverdin reductase, and the indicated concentrations of biliverdin and NADPH or NADH. The initial velocities were adopted for comparison of the reaction rates. *a*, With NADPH varied; *b*, with NADH varied.

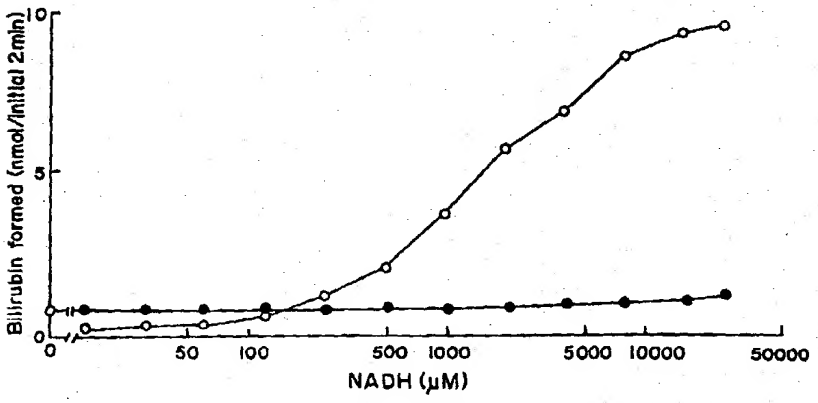

Fig. 6. Competitive inhibition of NADH-dependent biliverdin reductase reaction by NADPH. The assay mixtures contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 10 μM biliverdin, 1 mg/ml bovine serum albumin, 2 μg protein of purified splenic biliverdin reductase, and the indicated concentrations of NADH or both NADH and NADPH. ○, With NADH alone; ●, with the indicated concentrations of NADH plus 100 μM NADPH.

that for NADPH was about 3 μM, while in the reaction with NADH, K_m for biliverdin was 1–2 μM and that for NADH was 1.5–2 mM. It was found that the maximum velocity of the reaction with NADH was about twice that of the reaction with NADPH at pH 7.4.

It should be noted that the K_m value for NADPH is far smaller than that for NADH. Preference for NADPH in the biliverdin reductase reaction was confirmed by the experiments shown in Fig. 6. When NADH alone was employed as a reductant, the rate of reduction of biliverdin

increased with further addition of NADH, but when the reaction mixtures were supplemented with 100 μM NADPH, which appeared to be sufficient to give maximum activity in the NADPH-dependent reaction, the rates in the reaction systems containing both NADH and NADPH did not increase beyond the level which could be reached by employing NADPH alone; in other words, the observed increase in the rate of the reaction with NADH alone was suppressed almost completely by 100 μM NADPH, indicating that NADPH was preferentially utilized for the reduction of biliverdin under these reaction conditions. These data are consistent with the view that NADPH and NADH compete for the same binding site of a single enzyme protein. We have also observed in an independent experiment that reduction of biliverdin in the reaction system with 2 mM NADH was inhibited almost completely by the addition of as little as 5–20 μM $NADP^+$, whereas the extent of the inhibition by 2 mM NAD^+ was about 65%. On the other hand, the reduction of biliverdin in the reaction system with 100 μM NADPH was hardly affected by 2 mM NAD^+, while the reduction was inhibited by about 85% by the addition of 1 mM $NADP^+$. These observations also indicate that biliverdin reductase utilizes NADP-type pyridine nucleotides in preference to NAD-type pyridine nucleotides.

Stoichiometry of Biliverdin Reduction—In biliverdin reduction catalyzed by biliverdin reductase, 1 mol of reduced pyridine nucleotide was apparently consumed per mol of biliverdin reduced or per mol of bilirubin formed in both the NADPH and NADH systems, as shown in Table II.

Comparison of the Effectiveness of Four Biliverdin Isomers as Substrates—Four biliverdin isomers, *i.e.*, the IXα-, IXβ-, IXγ-, and IXδ-isomers, prepared according to Bonnet and McDonagh (*14*), were subjected to enzymic reduction with highly purified splenic biliverdin reductase. Since there is no reliable means to determine accurately the concentrations of the isomers other than IXα-biliverdin, the concentrations of the other isomers were estimated from the absorption at 650–670 nm, assuming that the three isomers have the same millimolar extinction coefficients at 650–670 nm as IXα-biliverdin at 670 nm, namely 15.0. Figure 7 shows the absorption spectra of the four biliverdin isomers used as substrates. The spectra of

TABLE II. Stoichiometry in the biliverdin reductase reaction. The assay mixture (final, 2 ml) contained 0.1 M potassium phosphate buffer (pH 7.4), 13 μM biliverdin, 1 mg/ml bovine serum albumin, 100 μM NAD(P)H, and 1.5 μg protein of purified splenic biliverdin reductase. Biliverdin was omitted in the control. The reaction was started by adding biliverdin after preincubation for 5 min at 37°C. At the indicated times, the difference spectra in the whole range of 340–700 nm were recorded. The amounts of bilirubin formed were determined as described in the text. The amounts of biliverdin lost were calculated from the decrease in absorbance at 670 nm, assuming the millimolar extinction coefficient for the decrement at 670 nm to be 13.0, since bilirubin also absorbed at 670 nm, giving a millimolar extinction coefficient of 2.0. The amounts of NAD(P)H consumed were calculated from the decrease in the absorbance at 340 nm, employing a millimolar extinction coefficient of 23.2 instead of 6.2 for the decrement at 340 nm, since the millimolar extinction coefficients of biliverdin and bilirubin at 340 nm were 25.0 and 8.0, respectively.

Reaction time (min)	Bilirubin formed (nmol)	Biliverdin lost (nmol)	NAD(P)H consumed (nmol)
Reaction with NADPH			
20	6.74	6.54	6.21
40	14.00	13.15	13.53
60	23.04	20.24	22.16
Reaction with NADH			
20	2.22	2.88	2.47
40	5.30	5.65	5.06
60	8.00	8.11	8.53

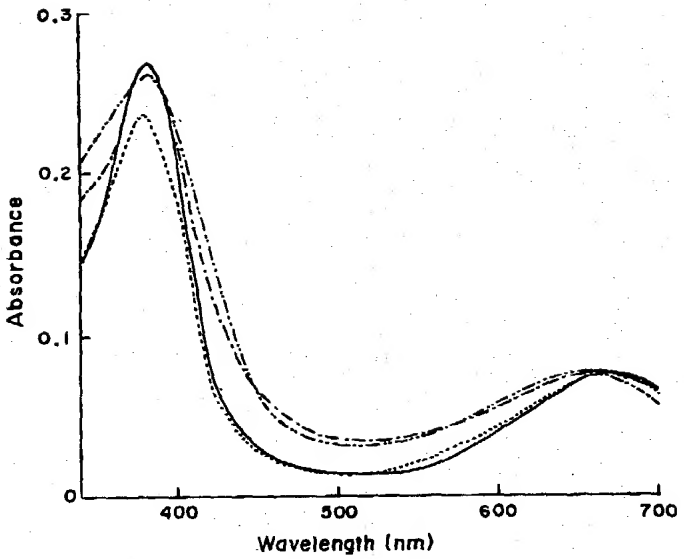

Fig. 7. The absorption spectra of the four biliverdin isomers in assay mixtures for the biliverdin reduction but without pyridine nucleotides. The mixtures contained, in 1.9 ml, 200 μmol of potassium phosphate buffer (pH 7.4), 2 mg of bovine serum albumin, 14 μg of purified splenic biliverdin reductase, and approximately 10 nmol of IXα (——), IXβ (—·—), IXγ (—··—), or IXδ-biliverdin (·····).

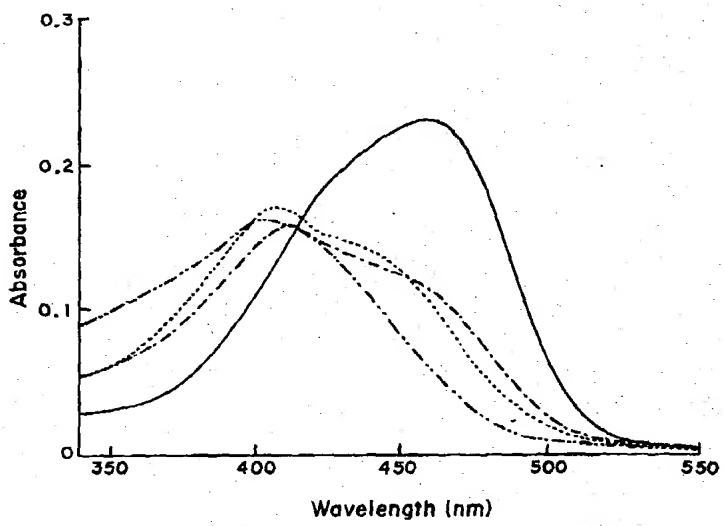

Fig. 8. The absorption spectra of the reaction products extracted into 3 ml of butanol from the reaction mixtures after completion of the reaction. The compositions of the reaction mixtures are given in the legend to Fig. 9. The reactions were started by the addition of 0.1 ml of NADPH (final, 100 μM). ——, IXα-isomer; —·—, IXβ-isomer; —··—, IXγ-isomer; ·····, IXδ-isomer.

the products of the reactions with the four isomers, which were extracted in butanol after completion of the reactions, are compared in Fig. 8. The spectral properties of the isomers shown in Figs. 7 and 8 are quite similar to those described by Blanckeart *et al.* (*16*). In the reaction with the α-isomer of biliverdin, two clear isosbestic points were observed at 408 nm and 525 nm; with the

β-isomer, they were at 398 nm and 518 nm; with the γ-isomer, they were at 388 nm and 492 nm; and with the δ-isomer, they were at 395 nm and 512 nm. Figure 9 shows the rates of reduction

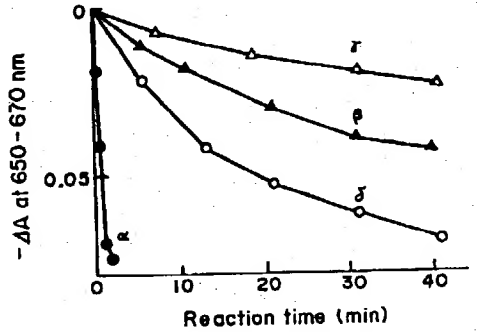

Fig. 9. Comparison of the reduction rates of the four biliverdin isomers. The assay mixture contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 1 mg/ml bovine serum albumin, 14 μg protein of purified splenic biliverdin reductase, and one of the four biliverdin isomers (indicated by α, β, γ, and δ in the figure). The final concentration of each biliverdin isomer was 5 μM.

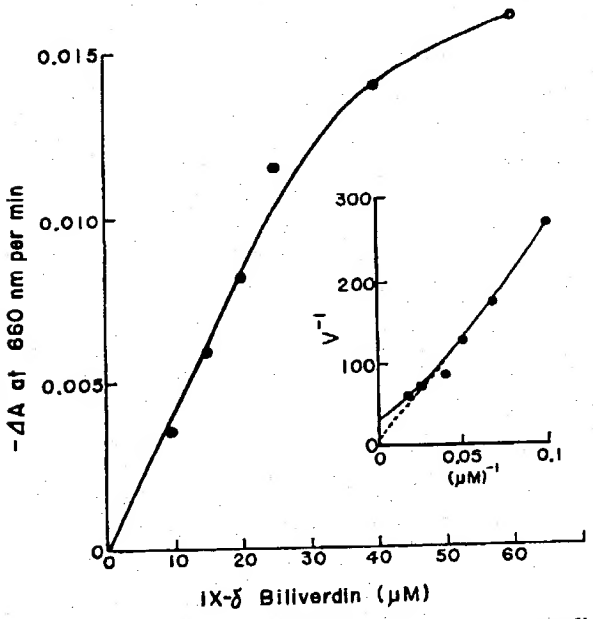

Fig. 10. Kinetics of reduction of IXδ biliverdin. Reaction mixtures contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 1 mg/ml bovine serum albumin, 14 μg of purified splenic biliverdin reductase, and the indicated concentrations of IXδ biliverdin. The reaction was started by the addition of NADPH (final 100 μM). *Inset*: double-reciprocal plot.

of the four biliverdin isomers in the NADPH-dependent reactions. Although the rates of reduction of the β-, γ-, and δ-isomers were extremely low as compared with that of the α-isomer, the initial rates were exactly proportional to the enzyme amounts used (data not shown). Taking the initial rate of reduction of the α-isomer as 100%, the relative initial rates of reduction of the β-, γ-, and δ-isomers were 4%, 2%, and 7%, respectively. The observed low rates of reduction of the unphysiological isomers are probably due to low affinities of these isomers for biliverdin reductase. In fact, as shown in Fig. 10, the half-saturation concentration of IXδ-biliverdin (apparent K_m value) were about 30 μM, which is more than 60 times that for IXα-biliverdin (0.3–0.5 μM) in the NADPH-dependent reaction. The double reciprocal plot of the data, however, was not linear, but was convex downwards, suggesting the occurrence of mild substrate inhibition for the reaction with the δ-isomer of biliverdin. It should also be noted that the maximum velocity of reduction of the δ-isomer, as estimated by extrapolation of the linear part of the double reciprocal plot, was apparently the same as the maximum velocity of reduction of the α-isomer under comparable experimental conditions, except for the biliverdin concentration.

Effects of Bovine and Human Serum Albumins —Previous investigators (*8*, *9*) had noticed that bovine serum albumin and human serum albumin enhanced the biliverdin reductase reaction. However, the relationships between albumin concentration and the extent of the stimulation reported for bovine serum albumin (*8*) and human serum albumin (*9*) were considerably different, so the ways in which these albumins exert their stimulative effects appear to be different. We therefore examined the effects of human serum albumin and bovine serum albumin on the reaction, employing the purified biliverdin reductase. Figure 11 shows the effects of increasing concentrations of albumins on the rate of reduction of biliverdin. In these experiments 10 μM biliverdin was used, as usual. Bovine serum albumin stimulated the reactions with both NADPH and NADH, although the degree of stimulation was considerably greater in the reaction with NADH. Human serum albumin also stimulated both reactions, but excess human serum albumin was rather inhibitory to both

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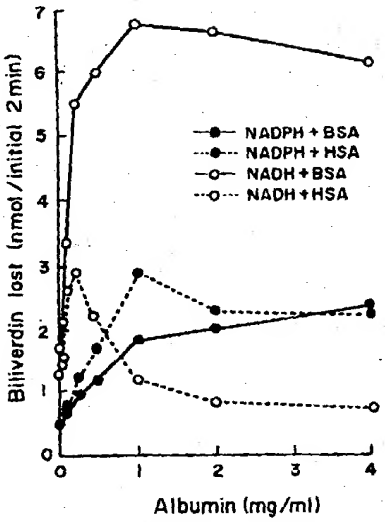

Fig. 11. Effects of bovine serum albumin (BSA) and human serum albumin (HSA) on the biliverdin reductase reaction. In this series of experiments, the reaction rate was measured in terms of the disappearance of biliverdin instead of bilirubin formation, since the spectrum of bilirubin is considerably different according to whether it is bound with albumin or not. The amount of biliverdin lost was calculated from the decrease in absorbance at 670 nm, assuming the millimolar extinction coefficient at 670 nm to be 13.0 for the decrement of biliverdin, as discussed in the text. In the experiments with human serum albumin, however, a slight correction to the millimolar extinction coefficient was made since human serum albumin had a slight metachromagic effect on biliverdin, as mentioned in the text. The reaction mixtures contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 10 μM biliverdin, 100 μM NADPH (or 2 mM NADH), 4 μg protein of purified splenic biliverdin reductase, and the indicated amounts of bovine or human serum albumin.

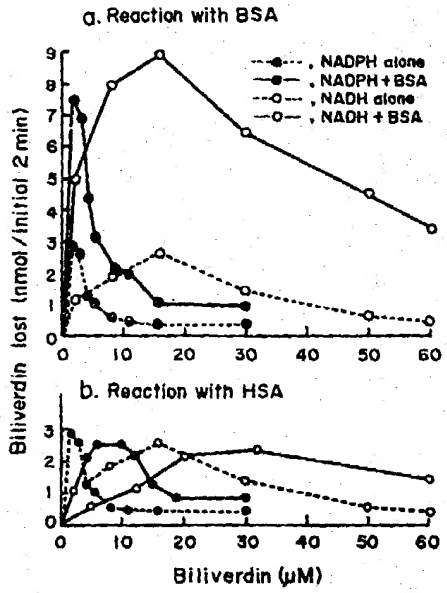

Fig. 12. Effects of addition of bovine and human serum albumins on the biliverdin reductase reaction at various biliverdin concentrations. The amounts of biliverdin reduced were calculated in the same way as in Fig. 11. *a*, The reaction mixture contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 100 μM NADPH (or 2 mM NADH), 4 μg protein of purified splenic biliverdin reductase, the indicated concentrations of biliverdin, and 2 mg of bovine serum albumin (BSA), when added. *b*, The compositions of the reaction mixtures as well as the meaning of the symbols were the same as in *a*, except that 2 mg of human serum albumin (HSA) was employed in place of BSA.

reactions. These results are similar to those reported by previous investigators (*8*, *9*).

Since the biliverdin concentration used in the above experiments was 10 μM, which was quite inhibitory to the reaction with NADPH (*cf.* Fig. 4), it is possible that albumin might have acted to reduce the inhibitory action of excess biliverdin. Therefore the effects of albumins at different concentrations of biliverdin were examined. As shown in Fig. 12, *a*, bovine serum albumin (1 mg/ml) enhanced the reaction to approximately the same degree at any biliverdin concentration tested in the NADPH system or the NADH system. In contrast, as shown in Fig. 12, *b*, human serum albumin (1 mg/ml) reduced the rate of the reaction at relatively low concentrations of biliverdin in the NADH or NADPH system, while at relatively higher concentrations of biliverdin, it apparently reduced the extent of the inhibition by excess biliverdin. However, with both NADPH and NADH, the maximum activities obtained in the presence of human serum albumin were similar to those observed without addition of albumin, indicating that the stimulatory effect of human serum albumin is far smaller than that of bovine serum albumin.

The observed stimulatory effect of human serum albumin could be accounted for by assuming that it binds biliverdin fairly tightly, reducing the effective concentration of biliverdin in the

reaction mixture. In accord with this view, we have observed that the addition of human serum albumin to biliverdin solution gave rise to a slight metachromagy of biliverdin (batho- and hyperchromic shift of the absorption maximum in the ultraviolet region and hyperchromic shift of the absorption maximum in the visible region). On the other hand, such metachromagy of biliverdin was not observable in the presence of bovine serum albumin, and this is in accord with the observation that bovine serum albumin did not significantly alter the pattern of inhibition by excess biliverdin. The possibility that bovine serum albumin might have stimulated the reaction by enhancing the removal of bilirubin as the product from the enzyme surface is also unlikely, since we observed in an independent experiment that the relative ratios of inhibition of the biliverdin reductase reaction by exogeneously added bilirubin were not substantially different whether the reaction mixtures contained bovine serum albumin or not (data not shown). Bovine serum albumin appears to stimulate the activity of biliverdin reductase in a rather direct manner through an as yet unknown mechanism.

Purification and Molecular Properties of Hepatic Biliverdin Reductase—Rat liver biliverdin reductase was purified by a modification of the procedures used for the purification of the enzyme from pig spleen. The results of a typical experiment are shown in Table III. Pooled rat livers (950 g) were homogenized with 4 volumes of 0.02 M potassium phosphate buffer (pH 7.4) containing 0.134 M KCl. The homogenate was successively centrifuged at 8,000 × *g* for 15 min and at 77,000 × *g* for 90 min, and the supernatant obtained was fractionated with solid ammonium sulfate between 0.35–0.7 saturations. The fractionated proteins were subjected successively to chromatographies on a CM-cellulose column (CM 32, 5 × 30 cm) and three separate hydroxyapatite columns (5 × 10 cm) as described for the purification of the splenic enzyme. Hepatic biliverdin reductase, however, was not adsorbed on hydroxyapatite columns which had been equilibrated with 10 mM potassium phosphate buffer, and therefore the flow-through fractions and washings were combined (hydroxyapatite fraction, 1,250 ml). The first Sephadex G-100 fraction was obtained as described in the previous section, and this fraction was loaded onto a DEAE-cellulose column (DE-32, 2.5 × 45 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 30 mM KCl. After washing the column with 200 ml of the same buffer,

TABLE III. Summary of the purification of biliverdin reductase from rat liver. The procedures are described in detail in the text. About 1 mg of protein was used for the assay of biliverdin reductase activity in the fractions from the 77,000 × *g* supernatant to the hydroxyapatite fraction.

Fraction	Total protein (mg)	Biliverdin reductase activity				Ratio of activities measured with NADH and with NADPH
		With NADPH (100 μM)		With NADH (2 mM)		
		Specific activity (unit/mg)	Total activity (unit)	Specific activity (unit/mg)	Total activity (unit)	
77,000 × *g* supernatant	79,800	0.042	3,350	0.183	14,600	4.3
Ammonium sulfate (35–70%)	68,600	0.048	3,290	0.250	17,200	5.2
CM-cellulose	55,700	0.060	3,340	0.302	16,800	5.0
Hydroxyapatite	34,000	0.076	2,580	0.412	14,000	5.4
1st Sephadex G-100	4,470	0.391	1,750	2.12	9,470	5.4
1st DEAE-cellulose	242	2.48	600	15.5	3,750	6.2
2nd DEAE-cellulose	143	3.28	468	19.6	2,800	6.0
QAE-Sephadex	34	7.12	244	51.2	1,760	7.2
Electrofocusing	—	—	81	—	560	6.9
2nd Sephadex G-100	4.2	8.39	35	60.4	254	7.2

biliverdin reductase was eluted with a linear gradient formed from 600 ml of the same buffer and 600 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 90 mM KCl. The most active fractions, which were eluted at about 35–40 mM KCl, were combined (1st DEAE-cellulose fraction, 370 ml) and diluted 3-fold with distilled water. The diluted solution was again charged onto a DEAE-cellulose column (DE-32, 2.5×23 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.4) containing 30 mM KCl, and the enzyme was eluted in the same manner as in the first DEAE-cellulose column chromatography, except that a 500 ml linear gradient was employed instead of 1,200 ml. The biliverdin reductase activity was again eluted at 35–40 mM KCl. The second DEAE-cellulose fraction (140 ml) was dialyzed against 10 mM potassium phosphate buffer (pH 7.4) containing 60 mM KCl and the dialyzed solution was adsorbed on a QAE-Sephadex column (1.5×30 cm) equilibrated with the same buffer. After rinsing, protein was eluted with a linear gradient formed from 150 ml of the rinsing buffer and 150 ml of 10 mM potassium phosphate buffer (pH 7.4) containing 250 mM KCl. The active fraction (QAE-Sephadex fraction, 110 ml) was concentrated by membrane filtration to about 15 ml and subjected to preparative electrofocusing (110 ml capacity, LKB) in the pH range of 4 to 6. The isoelectric point of the hepatic biliverdin reductase appeared to be 5.4. The electrofocusing fraction (15 ml) was further fractionated on a column of Sephadex G-100 (2.5×100 cm), using 50 mM potassium phosphate buffer (pH 7.4) as an eluant.

In the experiment shown in Table III, the purification was about 300-fold with an activity recovery of about 1.5%. The enzyme preparation finally obtained showed a distinct protein band with a molecular weight of 34,000 on SDS-gel electrophoresis, but there were also several minor protein bands on the gel. Further purification was unsuccessful due to instability of the enzyme. The purified enzyme preparation had a molecular weight of 34,000 as estimated by gel filtration on a Sephadex G-200 column (2.5×100 cm) (data not shown). The hepatic biliverdin reductase also appears to be a monomer protein with a molecular weight of 34,000. The purified hepatic enzyme could be stored at −20°C for several months without appreciable loss of activity.

It should be noted in Table III that, although the NADPH- and NADH-dependent biliverdin reductase activities were both purified concomitantly throughout the overall purification procedure, the extents of purification of the activities at individual steps were not always the same. Also, the ratio of the NADH-dependent and NADPH-dependent activities gradually changed from 4.3 to about 7 as the purification proceeded. The activity ratio of 7 is the same as that observed for the purified or the crude splenic enzyme. To investigate the possibility that the rat liver extract, but not the pig spleen extract, may contain some proteins which readily bind biliverdin, we added IXα-biliverdin to a 77,000×g supernatant of the liver homogenate and fractionated it directly on a Sephadex G-100 column. It was found that most of the pigment added was eluted at the void volume, while small portions were also eluted over all the fractions up to the bed volume. We took the void volume fraction from another 77,000×g supernatant to which biliverdin had not been added, and examined the effect of addition of this fraction on the NADH-dependent and NADPH-dependent activities of the purified biliverdin reductase. As shown in Fig. 13, the NADH-dependent activity decreased progressively with addition of the void volume fraction, whereas this fraction did not affect the NADPH-dependent activity under the

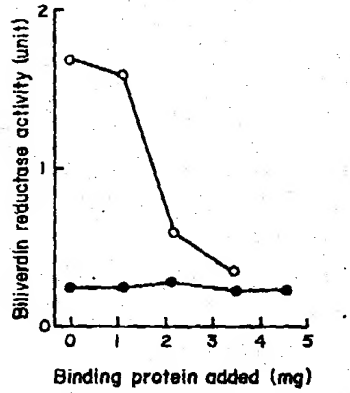

Fig. 13. Effect of biliverdin-binding protein(s) on the biliverdin reductase activity. Reaction mixtures contained, in a final volume of 2 ml, 100 mM potassium phosphate buffer (pH 7.4), 10 μM IXα-biliverdin, 1 mg/ml bovine serum albumin, the indicated amounts of biliverdin binding protein (the void volume fraction described in the text), and 100 μM NADPH (●) or 2 mM NADH (○).

experimental conditions employed. Apparently the enzyme preparations at earlier steps of the purification contained a biliverdin-adsorbing protein(s) and this acted to decrease the concentration of biliverdin available for the reaction. It is conceivable that only the NADH-dependent activity was affected by such a protein(s) since the NADH-dependent activity requires higher concentrations of biliverdin than the NADPH-dependent activity.

Catalytic Properties of the Hepatic Biliverdin Reductase—The purified hepatic biliverdin reductase was found to have catalytic properties very similar to those of the splenic enzyme. The hepatic biliverdin reductase purified from rat liver was active with both NADPH and NADH. K_m for NADPH was 3 μM and that for NADH was 1.5–2.0 mM. Essentially the same relation as depicted in Fig. 5 was also observed with the purified hepatic biliverdin reductase. K_m for IXα-biliverdin was 0.3 μM in the NADPH system and that in the NADH system was about 3 μM. These values are quite similar to those obtained for the reactions with splenic biliverdin reductase. Inhibition by excess biliverdin was also observable, and this inhibition was far more prominent in the reaction with NADPH than in that with NADH. The hepatic enzyme showed a very high activity with IXα-biliverdin as compared with the other isomers, like the splenic enzyme. All those observations indicate that the hepatic and splenic enzymes have essentially similar molecular and catalytic properties.

DISCUSSION

The present study with purified enzyme preparations from pig spleen and rat liver has shown that biliverdin reductase is a monomer protein with an apparent molecular weight of 34,000 and that a single enzyme is responsible for both the NADPH-dependent and NADH-dependent reactions, resolving the previous controversy (*8*, *9*) with respect to the hydrogen donor in the enzyme-catalyzed reduction of biliverdin.

The soluble fraction of rat liver contained a protein(s) which readily binds IXα-biliverdin. It is well known that the soluble liver fraction contains organic anion binding proteins such as ligandin (*21*, *22*) and Z protein (*22*, *23*), which preferentially bind bilirubin, bromsulfophthalein, indocyanine green, fatty acids, and others. The biliverdin binding protein(s) found in the present study may have a larger molecular weight than ligandin (M.W. 44,000 (*24*) or Z-protein (M.W. 12,000 (*23*, *25*)) since the biliverdin-binding protein was eluted at the void volume of a column of Sephadex G-100. No attempt was made to further characterize this protein.

It is worth noting that although biliverdin reductase was active with both NADH and NADPH, there were considerable differences in kinetic properties between the reactions with NADH and NADPH. Particularly significant is the observation that the inhibition by excess biliverdin was far more marked in the reaction with NADPH than with NADH. O'Carra and Colleran (*11*) also observed similar substrate inhibition and interpreted this as being similar to the type of abortive complex mechanism known to be responsible for substrate inhibition in some other pyridine nucleotide-linked dehydrogenase — notably lactate dehydrogenase (*26*). Namely, in the biliverdin reductase reaction, an inactive ternary complex consisting of enzyme, biliverdin and oxidized pyridine nucleotide may be readily formed, especially in the reaction with NADPH (*11*), because NADP-type pyridine nucleotides have higher affinity for biliverdin reductase compared with the NAD-type ones.

We confirmed, using the highly purified enzymes from pig spleen and rat liver, that biliverdin reductase has a much higher affinity for the α-isomer of biliverdin than for the other three types of isomers, and that the enzyme could reduce the α-isomer much faster than the other unphysiological isomers. This is important in relation to the physiological role of biliverdin reductase, since the microsomal heme oxygenase has been shown to yield almost exclusively IXα-biliverdin (*2*). Colleran and O'Carra (*10*, *11*) reported that the γ-isomer of biliverdin could not serve as a substrate when tested with the crude extract of guinea pig liver. With the purified pig spleen enzyme, however, we found that the γ-isomer could be reduced by the biliverdin reductase system, although the rate of reduction was extremely low, as shown in Fig. 9. On the other hand, the dimethylester of IXα-biliverdin was not a substrate of the purified biliverdin reductase

preparations (data not shown), in agreement with the observation of Colleran and O'Carra with the crude enzyme preparation (*10*, *11*). Free propionate side chain may be an essential requirement for biliverdin as a substrate, and the factor determining the reactivity of the individual biliverdin isomers may be the position of the propionate side chains on the tetrapyrroles, as suggested originally by O'Carra and Colleran (*11*).

It is well documented that NADP is predominantly in the reduced form in the cytoplasm, whereas NAD is predominantly in the oxidized form (*27*). The approximate concentrations of pyridine nucleotides in the liver cytosol fraction have been reported to be as follows: 0.12 mM for NADPH, 0.035 mM for $NADP^+$, 0.07 mM for NADH, and 0.5 mM for NAD^+ (*28*). Taking into account these values as well as the K_m values for NADH and NADPH in the biliverdin reductase reaction, it appears that the enzyme-catalyzed conversion of biliverdin to bilirubin in animal tissues under physiological conditions may depend almost entirely on NADPH.

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